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13. ABSTRACT (Maximum 200 Words)

BRCA1 is strongly associated with the breast cancer. BRCA1 associates with numerous proteins that repair DNA damage. Fanconi Anemia (FA) is a rare autosomal recessive disorder. It has been shown that BRCA1 regulates one of FA proteins, called FANCD2, by a process called ubiquitination. However, exactly how the FA proteins and BRCA1 interact to regulate DNA damage repair obscure. In this project, we hypothesize that BRCA1 ubiquitination of FANCD2 is affected by association with the FANCA protein complex and by association with DNA damage when embedded in chromatin. Specific aims are that (1) does BRCA1 monoubiquitinate FANCD2 in vivo using purified ubiquitination factors? (2) Do embedding FA proteins in chromatin affect their function as ubiquitination substrates? (3) Is the ubiquitination of FA proteins by BRCA1 affected by binding to damaged DNA? During the first year of grant, we purified the FANCD2, FANCA and BRCA1/BARD1 from baculovirus-infected insect cells and we identified that BRCA1 could ubiquitinated FAND2, dependent on the E2 enzymes. Also, we found direct DNA binding activity of the FANCD2 protein. Now we are trying to know the function of FANCD2 and modified FANCD2 (ubiquitinated FANCD2) on DNA or chromatin. Finally, we are trying to understand the relationship between BRCA1 and FANCD2 in the DNA damage repair pathway by keeping this project.

14. SUBJECT TERMS
Breast cancer, BRCA1, FA, FANCD2, ubiquitination, deubiquitination

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Annual Summary Report
Award Number W81XWH-04-1-0462
PI: Woo-Hyun Park, Ph.D.

Introduction

In this project, we study the inter-relationship of BRCA1 and FANCD2, and we have made considerable progress toward completion of the specific aims. BRCA1 is the breast-specific tumor suppressor protein 1. FANCD2 is the Fanconi Anemia protein D2. Both proteins execute vital functions in the repair of DNA damage, but how this occurs is unknown. In this project, we hypothesize that BRCA1-dependent ubiquitination activity modifies FANCD2, and the resulting change in the FANCD2 causes a change in its activity and affects the DNA repair process.

Specific aims in our project are
(1) Does BRCA1 monoubiquitinate FANCD2 in vivo using purified ubiquitination factors?
(2) Do embedding FA proteins in chromatin affect their function as ubiquitination substrates?
(3) Is the ubiquitination of FA proteins by BRCA1 affected by binding to damaged DNA?

Task 1: Purification of FANCD2, BRCA1, and associated factors, and testing whether BRCA1 ubiquitinates FANCD2.

Status: Completed

We purified the FANCA, FANCD2, BRCA1, and BARD1 proteins from recombinant baculovirus infected insect cells exactly as originally proposed (Figure. 1).

In FT E

mock FANCD2

183 ► ◄ D2
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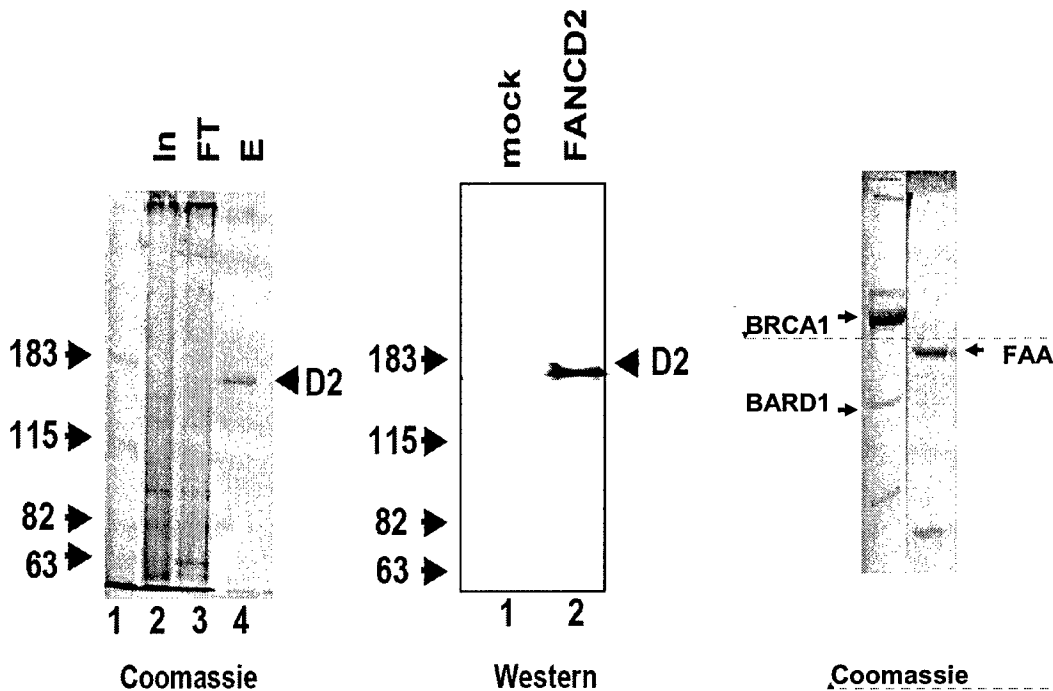63 ►
1 2
Western

BRCA1 ►
BARD1 ►
◄ FAA
Coomassie

Figure 1. FANCD2, FANCA and BRCA1

Purified proteins were subjected to SDS-PAGE analysis. FANCD2 protein migrated at 160 kD (left panel), consistent with its predicted mass, and immunoblot analysis revealed that this polypeptide was indeed FANCD2 (middle panel). FANCA and BRCA1/BARD1 were also purified from baculovirus-infected insect cells and analyzed by SDS-PAGE and Coomassie stain.

Next, we undertook the in vitro ubiquitination assay to ascertain ubiquitination system dependent on the BRCA1/BARD1. Consistent with our hypothesis, we found that BRCA1 ubiquitinated FANCD2 dependent on the specific E2 ubiquitin conjugating enzyme (Ubc; Figure. 2). BRCA1/BARD1 (B/B)

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proteins ubiquitinated the FANCD2 protein dependent on UBCH5a or Ubc6. However, to our surprise, BRCA1 was not required for the ubiquitination of FANCD2 in the presence of UbcH5c. In this last case, the ubiquitination was independent of the E3 ligase. FANCA had minimal effect on the ubiquitination reaction (not shown). We conclude that the BRCA1 has some important roles in the ubiquitination of FANCD2 in response to the DNA damage, but it is not essential.

E2 : Ubc7   Ubc3   UbcH5a UbcH5c   Ubc6

B/B: +   +   +   +   +

← ub-FAD2
← FAD2

Figure 2. FANCD2 ubiquitination by B/B.

Task 2: Is ubiquitination of FANCD2 by BRCA1 affected by embedding FANCD2 in chromatin?
Status: in progress.

Due to results in Task 3, this specific aim will be completed after aim 3.

Task 3: Is the ubiquitination of FA proteins by BRCA1 affected by binding to damaged DNA?

Status: In progress.

We have made considerable progress in this aim. We hypothesized that the FANCD2 protein binds to DNA in a structure-specific fashion. The structures would exist at sites of DNA damage. Since Holliday junction DNA is a potential intermediate in the homologous recombination pathway, we tested this structure first. In an electrophoretic mobility shift assay (EMSA), full-length FANCD2 retarded the mobility of a 125-bp Holliday junction DNA probe (four 30 bp arms; Figure 3). Three different recombinant protein preparations from baculovirus-infected cells were analyzed. FANCD2 was compared to FANCA and to a mock purification from empty baculovirus-infected cells. FANCD2 (40 nM) bound to the Holliday junction DNA and migrated in a diffuse shift that formed a band

Probe: Holliday junction

Mock B/B FAA FAD2

below the origin of the gel. As a positive control, the BRCA1/BARD1 could bind to the Holliday junction DNA probe well in our system. FANCA protein did not bind to the Holliday junction DNA probe at the same concentration of FANCD2 protein. The shifted bands in the reaction with FANCA were also present in the mock purification from the cells infected with empty virus indicating that these complexes were low level, nonspecific contaminants.

Figure 3. FANCD2 binds DNA.

FANCD2 was included in DNA binding reactions at different concentrations (Figure. 4). We observed diffuse bands that migrated progressively slower in reactions with higher FANCD2 concentrations. This finding was most consistent with the interpretation that the protein-DNA complex contained multiple FANCD2 molecules. Since the stoichiometry of DNA binding could not be determined, we did not determine a K_d for the complex. Instead, we estimated binding affinity by the concentration of FANCD2 protein that bound half of the probe. Approximately half of the Holliday junction DNA probe was bound in reactions containing 15 nM FANCD2.

The specificity of DNA binding was determined using various probes and DNA competitors. FANCD2 bound to 65-bp double-stranded linear DNA (Figure. 4) and to a 65 bp Y shaped DNA (data not shown). When using a 65 bp linear DNA probe, 50% of the probe was bound at about 20 nM
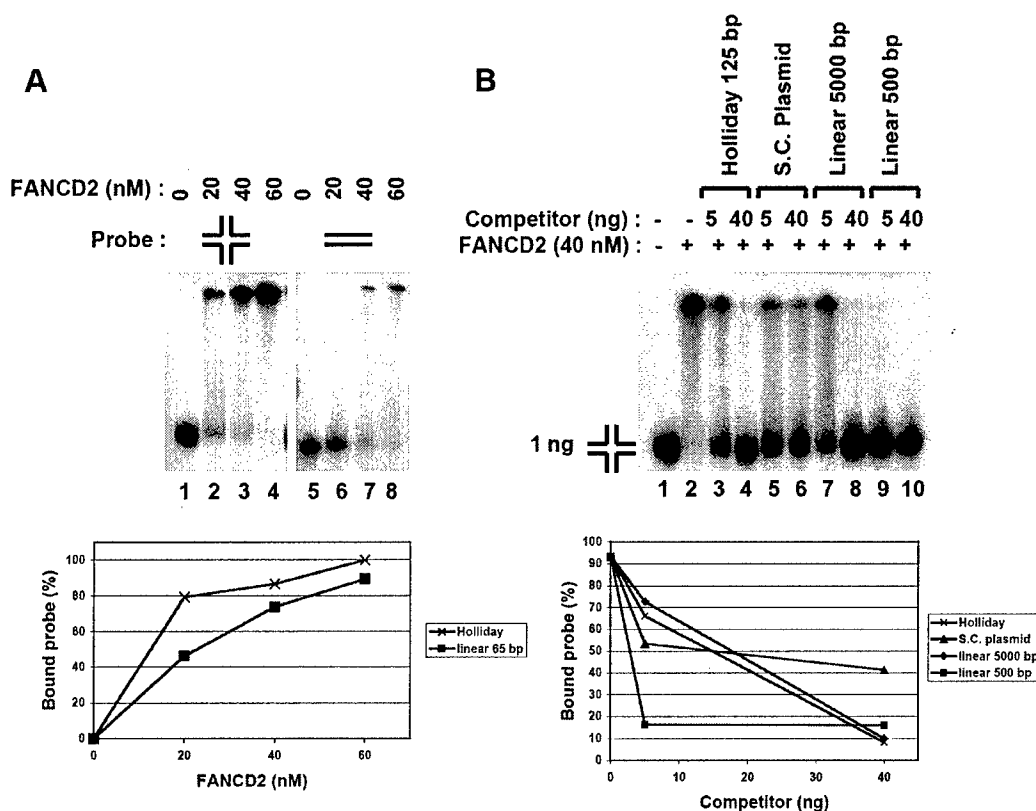
Figure 4. FANCD2 binds to various DAN forms

FANCD2, indicating that the binding affinity of FANCD2 to linear 65 bp was modestly decreased relative to the 125 bp Holliday junction probe. By competition analysis (Figure 4B), we found that FANCD2 bound to Holliday junctions and to DNA ends.   Surprisingly, ends on short DNA molecules

did not bind to FANCD2 (not shown), but DNAs containing an end and about 500 bp DNA were effective in binding.

Plans for this task will include the analysis of how ubiquitination by BRCA1 affects the DNA binding activity of FANCD2.

Key Research Accomplishments

1. Purification of FANCA and FANCD2 from insect cells.
2. Making the FANCA and FANCD2 stable cell lines.
3. Establishment of in vitro ubiquitination using the BRCA1/BARD, E1, E2, ubiquitin, FANCD2
4. Understanding that BRCA1 is an important for FANCD2 function but is not essential for ubiquitination of FANCD2
5. Finding the direct DNA binding activity of FANCD2 protein

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... [12]

Reportable Outcomes

1. The manuscript entitled "Direct DNA binding activity of the Fanconi Anemia D2 protein" has been accepted for publication in J Biol Chem.

Conclusions

During the first year of the award from the Department of Defense Breast Cancer Research Program, we have established the in vitro ubiquitination assay using the BRCA1/BARD1, E1, E2 and FANCD2 of substrate. We found that BRCA1 ubiquitinated the FANCD2 protein dependent on the E2 enzymes (UBCH5a and UBCH6) in vitro. However, from published data, another enzyme is required for the ubiquitination of FAND2 in a cell. Surprisingly, the ubiquitination of FANCD2 in vitro could occur independent of any E3 ligase. FANCA did not stimulate or repress the ubiquitination of FANCD2. Taken together, although BRCA1 has some important roles in the ubiquitination of FANCD2 in response to the DNA damage, it is not essential. Since FANCD2 functions in the nucleus and regulates the repair of DNA damage, we tested whether the FANCD2 protein binds to DNA. This study reveals that the FANCD2 protein binds to Holliday junction DNA and to DNA ends. This is the first biochemical activity identified for this key protein in the Fanconi Anemia pathway. This activity is consistent with a role for the FANCD2 protein in the repair of double stranded DNA breaks. However, it is unclear how phosphorylation and ubiquitination of FANCD2 regulate this activity and more work will be required to determine how the FANCD2 protein functions in the repair of DNA damage. Therefore, in future work we will test whether the FANCD2 protein functions in the repair of DNA damage. In the mean while, we also try to know the BRCA1 function in regulation of FANCD2 or cooperation with FANCD2 in the DNA damage response, especially breast cancer cell lines.

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Appendices

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Exactly How BRCA1 mutation causes breast cancer is unclear. Evidence suggests that the repair genomic damage is important, and BRCA1 is known to modify the FANCD2 protein. This basic research project will identify a key regulatory function of BRCA1 and will identify an important pathway, which is altered in breast cells that acquire BRCA1 mutations. Thus, the project will reveal clues that are relevant for the etiology of breast cancer.

shuttle vector, pFB1 or in the retrovirus shuttle vector, pBabe. We made the baculovirus having FANCA and FANCD2 genes using the insect cells and made the retrovirus having FANCA and FANCD2 genes using the 293T cells. We succeed in purification of full-length FANCA and FANCD2 from the infected cells using the IgG sepharose and TEV protease (Fig. 1.). Also, we could purify the BRCA1/BARD1 complexes from insect cells

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Similar to as described above for the baculovirus, we observed the expression of the exogenous FANCD2 and FANCA protein in the 293T packaging cell for retrovirus. (Fig. 2.). Also, we could make the stable cell lines (Hela and MCF 10A(Breast Cell)) expressing the FANCD2 and FANCA proteins and observe the expression of those proteins. However, when we purified the FANCA and FANCD2 complexes from Hela and MCF10 cells, we could not observe other FANC proteins such as FANCC, FANCF, FANCE and FANCG. The obstacle to see other FANC proteins in FANCA complexes maybe results from (1) low expression of FANCA protein in Hela cells, (2) low amount of FANCA complexes from cells and (3) using wrong biochemical buffers during immunoprecipitation and washing etc. We hold this task now.

Figure 1. Purification of FANCD2, FANCA, and BRCA1/BARD1 from baculovirus-infected insect cells.

E2 :   **Ubc7**   **Ubc3**   **UbcH5a** **UbcH5c**   **Ubc6**

B/B: **+**   **-**   **+**   **-**   **+**   **-**   **+**   **-**   **+**   **-**

← ub-FAD2
← FAD2

, similar to the published report

Even, we could see the ubiquitination of FANCD2 without the BRCA1

stimulates or represses the ubiquitination of FANCD2, we put together the FANCA, FANCD2 and BRCA1 into the test tube. There was no big difference of efficiency of ubiquitination by FANCA

We tested the specificity of FANCD2 for different forms of DNA using competition reactions. The probe in each reaction was 1 ng of the 125 bp Holliday junction DNA. A five-fold excess of self competitor resulted in a 35% reduction of the bound probe (Fig. 5). A 40-fold excess of self competitor resulted in nearly complete competition (lane 4). Supercoiled plasmid DNA competed modestly with the Holliday junction probe for binding to FANCD2 (Figure 5B, lanes 5, 6). By contrast, when this same plasmid was cleaved at a single site generating an approximately 5000 bp linear DNA, the level of competition was similar to the self competition (Figure 5B, lanes 7, 8). Since the principal difference between the supercoiled plasmid and the linear DNA was the presence of DNA ends, we tested whether a higher concentration of DNA ends bound to FANCD2 with higher affinity. Cutting this plasmid at a total of nine sites (average size of the linear DNA was approximately 500 bp) made this dsDNA the most efficient competitor tested for FANCD2 binding. Nearly complete competition for the Holliday junction probe was observed with a five-fold excess of the 500 bp linear DNA (Figure 5B, lane 9). In lanes 5-10, the competitor DNAs were all derived from the same plasmid with the only difference being the number of ends: 0, 2, or 18. These results supported the notion that the FANCD2 binds to DNA ends.

In most of the experiments in Figures 4 and 5, the probe was Holliday junction DNA. FANCD2 binding to the Holliday junction DNA could be due to binding to ends (four ends per molecule) or due to binding to the internal part of the Holliday junction. We directly compared which of these DNA structures bound to FANCD2 with higher specificity. The competitor DNAs were normalized by the moles of DNA ends of unlabeled Holliday junction DNA or 65 bp linear duplex DNA included in reactions with Holliday junction probe. The linear duplex DNA was equivalent in length to two arms of the Holliday junction probe. In this experiment, 1 ng of Holliday junction probe was used; thus, the concentration of the probe was 1.2 nM, but since it had four ends, the concentration of dsDNA ends was about 5 nM. Significant competition for DNA binding by FANCD2 was apparent when reactions contained 25 nM Holliday junction ends (5 ng DNA; Figure 6, lane 3), but there was very little competition for binding when reactions contained 200 nM 65 bp duplex DNA ends (lane 10). This result suggested that for DNAs of similar size, FANCD2 bound to the Holliday junction DNA with higher affinity than it bound to DNA ends. An alternative model for the specificity was that the ends of the Holliday junction DNA were closely juxtaposed and favored binding of the FANCD2 when compared to the linear DNA. Since FANCD2 bound to both, DNA ends and Holliday junctions, the specificity of the DNA binding activity was consistent with a role for FANCD2 in the repair of double stranded DNA damage.

Fig. 6

Not all linear DNA molecules were equivalent for binding to FANCD2. When comparing the competition for binding to the Holliday junction probe, the linear 500 bp DNA was the most effective competitor (complete competition with about 3 nM ends; Figure 5B, lane 9), while the 65 bp linear DNA did not compete (Figure 6, lanes 7-10). Since the protein-DNA complex was observed as a diffuse band that migrated with progressively slower speed as more FANCD2 was included in reactions, we infer that FANCD2 binds to DNA as a multimer. The competition data were most compatible with the model that the FANCD2 nucleated its binding on the DNA end, and the FANCD2 then spread along the length of the DNA. Thus, when comparing the three linear DNAs tested for binding to FANCD2, the 5000 bp linear DNA had too low a concentration of ends, and the 65 bp linear DNA was too short, but the 500 bp linear DNA had the best balance of DNA ends and length.

Fig. 7.

The carboxy-terminus of the FANCD2, encoded by exon 44, is highly conserved among homologues in other eukaryotic species and therefore this domain may have an important cellular function required for MMC resistance. This domain is highly acidic, containing 12 acidic residues

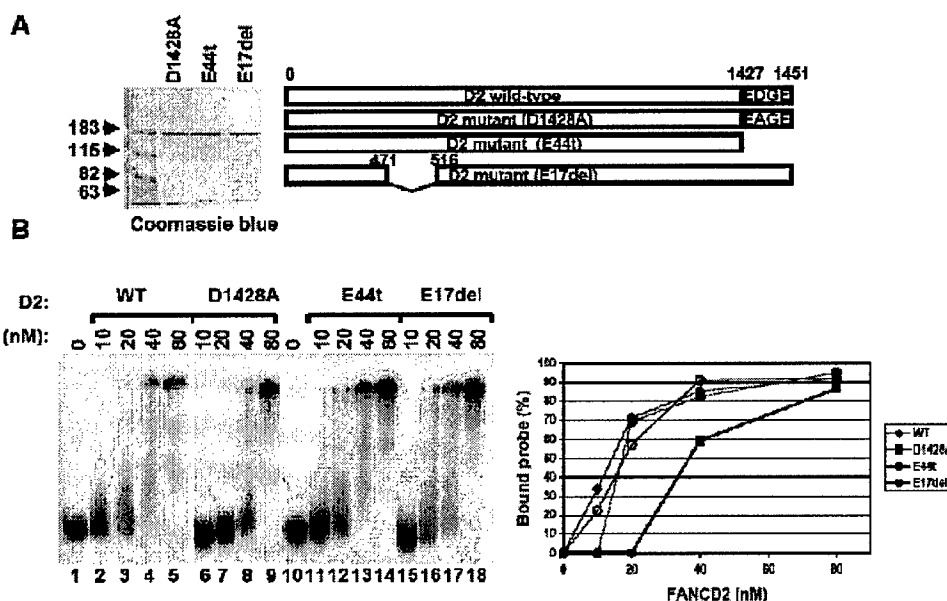out of 24 residues and is referred to as the "EDGE" domain. We purified a FANCD2 mutant, in which the first aspartic acid residue encoded in exon 44 was converted to alanine (D1428A). In addition, we truncated the acidic domain of FANCD2 (E44t) by expressing a fusion protein, which encodes no exon 44. Complementation studies reveal that expression of these mutants in FANCD2-deficient cells does not correct the FA phenotype. A Fanconi Anemia patient-derived mutation, which has no exon 17, was also expressed and purified (E17del). All three mutant FANCD2 proteins were expressed and purified similarly as was the wild-type protein (Figure 7A). Each of these protein preparations was analyzed for binding to Holliday junction DNA (Figure 7B). Each mutant form of FANCD2 bound to the DNA with an affinity similar to wild type protein. Removal of an acidic domain, as in the E44t FANCD2, would be predicted to increase affinity of a protein for DNA, but clearly, the magnitude of the effect was very small. The FANCD2 mutant D1428A did bind less avidly to the probe. While wild-type, E44t, and E17del FANCD2 each bound to 50% of the Holliday junction probe at approximately 15 nM protein, the D1428A mutant bound to 50% of the probe at about 40 nM (Figure 7B). This decrease in affinity of the mutant FANCD2-D1428A for binding DNA was small, and not seen with the E44t truncated protein, in which D1428 was deleted. Further, the patterns of the bands on the gel were similar when comparing the D1428A mutant with the wild-type FANCD2. We thus conclude that these mutations, which are associated with a Fanconi Anemia phenotype, do not significantly affect DNA binding properties of the FANCD2 protein.

Fig. 8.

In an effort to map the DNA
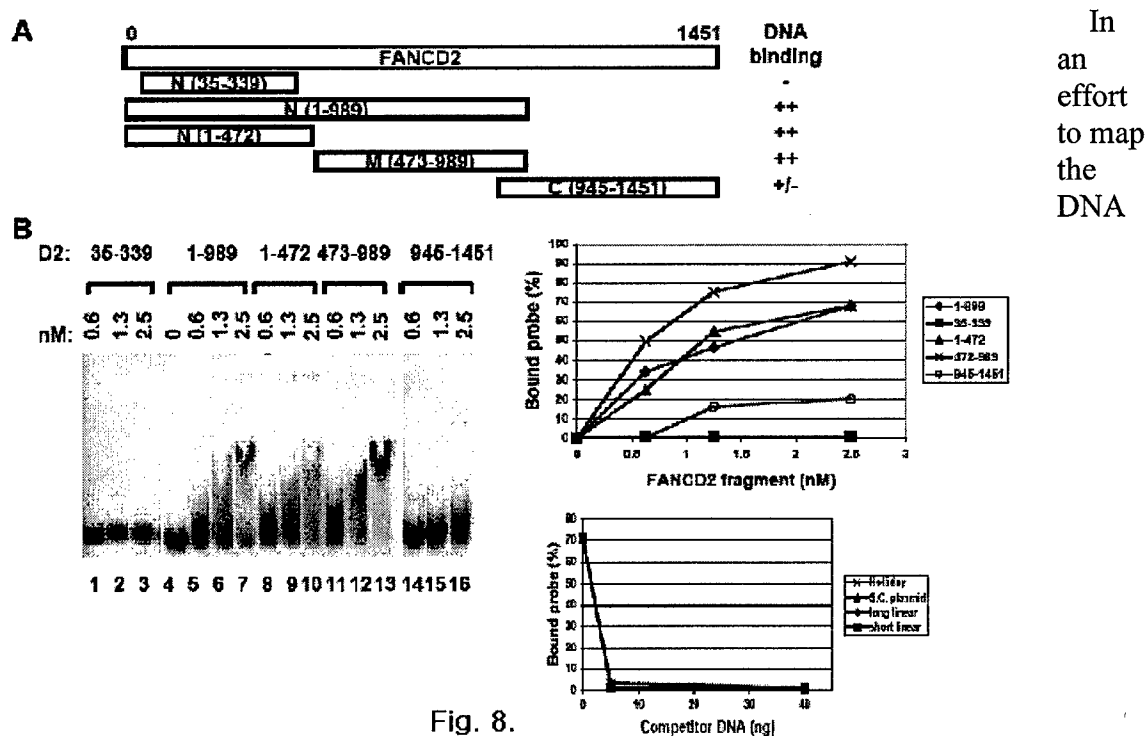
binding domain of FANCD2, we expressed and purified fragments of the protein. Data from Figure 7 revealed that a 44 amino acid domain from 472-515 was dispensable for DNA binding in the E17del mutant, and the 24 amino acid deletion from 1427-1451 in the E44t mutant was also competent for binding Holliday junction DNA. We expressed a

number of FANCD2 protein fragments, but in all cases the level of expression was low, and many of the different fragments were insoluble under nondenaturing conditions (data not shown). We did succeed in purifying the following FANCD2 fragments: 35-339 fused to GST; 1-989; 1-472; 473-989; and 945-1451. The protein fragments containing sequences 1-989, 1-472, and 473-989 all bound to the Holliday junction probes at lower concentration than did the full length (Figure 8), suggesting an increased affinity for the DNA. However, competition analysis revealed that all FANCD2 fragments bound to DNA in a nonspecific fashion (Figure 8). No identifiable DNA binding motif is revealed from the protein sequence. We conclude from these analyses that, with the exception of small deletions as in Figure 7, nearly the full length of FANCD2 is required for DNA binding activity. Taken together about the binding data, this study reveals that the FANCD2 protein binds to Holliday junction DNA and to DNA ends. This is the first biochemical activity identified for this key protein in the Fanconi Anemia pathway. This activity is consistent with a role for the FANCD2 protein in the repair of double stranded DNA breaks. However, it is unclear how phosphorylation and ubiquitination of FANCD2 regulate this activity and more work will be required to determine how the FANCD2 protein functions in the repair of DNA damage.

Therefore, we will try to know the FANCD2 protein functions in the repair of DNA damage. In the mean while, we also try to know the BRCA1 function in regulation of FANCD2 or cooperation with FANCD2 in the DNA damage response, especially breast cancer cell lines.

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